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THE ROLE OF GLYCOLYSIS AND HEXOSE MONOPHOSPHATE PATHWAY IN THE HYPOXIC TOXICITY OF MISONIDAZOLE

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The metabolic activation of misonidazole (MISO) and its effects on the hexose monophosphate pathway (HMP) and clonogenicity were studied in hypoxic EMT6/Ro, wildtype Chinese hamster ovary (CHO) and mutant CHO cells deficient in glucose-6-phosphate dehydrogenase. In all three cell lines metabolic activation of MISO, as indicated by the binding of ^{14}C-MISO to the acid-insoluble fraction of these cells, was increased by the presence of glucose. In EMT6/Ro cells and wildtype CHO cells, MISO caused a significant stimulation of the activity of the HMP while in the mutant CHO cells no HMP activity was measurable, even in the presence of MISO. Loss of clonogenicity induced by MISO occurred markedly earlier in EMT6/Ro cells than in the CHO cells. In the latter cells, however, only a small difference was observed between the wildtype and mutant cell line. From these results it is concluded that not only the HMP but also glycolysis and other, glucose-independent, metabolic pathways are able to provide electrons for the reductive activation of MISO and hence contribute to the hypoxic toxicity of this compound.

KEY WORDS: Cancer therapy, glycolysis, hexose monophosphate pathway, hypoxia, misonidazole.

INTRODUCTION

Misonidazole (1-(2-nitro-1-imidazolyl)-3-methoxy-2-propanol) (MISO) is currently under clinical trials in the chemo- and radiotherapy of tumors. It is selectively toxic against hypoxic cells, a characteristic which has been attributed to its reductive activation to reactive intermediates such as the nitro radical anion and the nitroso and hydroxylamine derivative (Fig. 1).[1–6] Enzymes described to catalyze this activation either by one- or two-electron reduction are the liver flavoenzymes cytochrome P-450 reductase, xanthine oxidase and DT-diaphorase and in tumor cells a microsomal enzyme described as NADPH-cytochrome c reductase.[1–6] The reaction of the first electron reduced form, the nitro radical anion, with oxygen to yield O_2^- and the parent nitro compound,[7] is considered responsible for the inhibitory effect of oxygen on the reductive activation and, thereby on the cell toxicity of this nitro compound. Products of its reductive metabolism thus far isolated and characterised are the six-electron reduced amino derivative and glyoxal, a decay product of the four-electron reduced hydroxylamine.[1,2,8,9] It is not known which of the reactive intermediates described above is the primary mediator of the hypoxic toxicity. Likewise it is unknown what the primary cellular damage is. Since reaction of intermediates of the reductive

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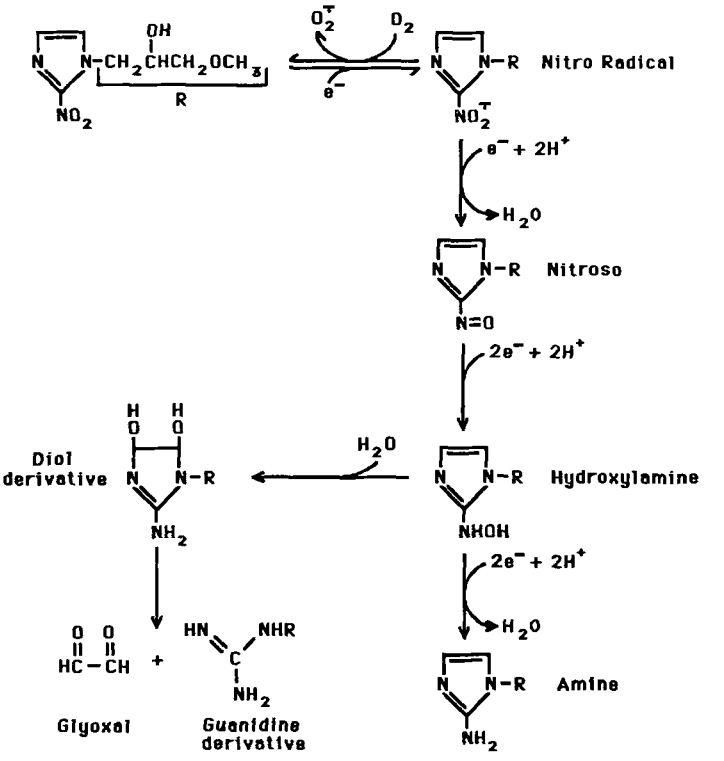

FIGURE 1 Scheme of the possible pathways of the reductive metabolism of misonidazole (MISO).

metabolism of MISO with protein-SH and nonprotein-SH, mainly GSH, have been reported, inactivation of cellular enzymes by reaction with their essential SH groups may be one cause of MISO cytotoxicity.[10] On the other hand, other damage such as DNA single strand breaks and the formation of DNA adducts have been described and may also account for the hypoxic toxicity of MISO and related nitro compounds.[11,12]

It is generally assumed that the electrons for the reductive activation of MISO are provided by the hexose monophosphate pathway (HMP) in form of NADPH. In the present paper, however, evidence will be presented that metabolic pathways other than the HMP may also provide reducing equivalents for the activation of MISO and thereby also contribute to the hypoxic toxicity of this nitroimidazole.

MATERIALS AND METHODS

BME (Eagles Basal Medium) and F-10 medium were purchased from Grand Island Biological Co. (Grand Island, NY). Trypsin was from Worthington Biochemical Corp. Freehold, NJ), Scintiverse from Fisher Scientific Company (Boston, MA), and

^{14}C-1-glucose from Research Products Int. Corp., (Mount Prospect, Illinois). Sera were from Flow Laboratories, Inc. (McLean, VA).

Misonidazole (MISO) and ^{14}C-MISO, labeled at the C-2 position of the imidazole ring were a gift from the Drug Synthesis and Chemistry Branch of the NIH. Mutant Chinese hamster ovary (CHO) cells deficient in glucose-6-phosphate dehydrogenase and their parent wildtype CHO cells were generously provided by Dr. T. Stamato of the Wistar Institute (Philadelphia, Pennsylvania).

EMT6/Ro cells were cultured in BME and CHO cells were grown in F-10 medium. Both media were supplemented with 15% (v/v) fetal bovine serum and contained 0.1 mg/ml streptomycin and 96 units/ml penicillin. The cells were grown in an atmosphere of 3% CO_2/97% air for EMT6/Ro cells and 5% CO_2/95% air for CHO cells as previously described.[13] For these studies, exponential cell cultures were dissociated with 0.01% trypsin for 10 min.

The incubations were performed at 37°C in 12 ml-incubation vessels. The cells and MISO in the respective media were deaerated separately by gassing with either 3% CO_2/97% N_2 (EMT6/Ro cells) or 5% CO_2/95% N_2 (CHO cells) for 1.5 h. After deaeration the cells were added to the MISO and the vials tightly sealed. The final concentrations were 10^6 cells/ml and 5 mM MISO. The total volume of the incubation mixture was 10 ml.

For the determination of the clonogenic survival, the cells were seeded into plastic culture dishes at concentrations to give 50 colonies. Triplicates of two dilutions were set for each experimental point determined. After 11 days the plates were stained with 0.03% methylene blue and colonies of greater than 50 cells were counted.

The activity of the hexose monophosphate pathway was measured by the formation of $^{14}CO_2$ from 6 mM ^{14}C-1-glucose (0.1 μCi/ml). $^{14}CO_2$ was released from the incubation medium by acidification with 0.2 ml 6 M HCl. It was trapped in 1 M KOH by flushing air through the vials for 30 min. After flushing, an aliquot (1 ml) of the KOH was counted in 10 ml scintillation fluid (Scintiverse) to determine the amount of $^{14}CO_2$ trapped in it.

Binding of ^{14}C-MISO (specific activity 0.2 mCi/mmol) to the acid-insoluble fraction of the cells was determined as follows: After different times of incubation, 1 ml of the incubation mixture was removed and the cells were spun down. The pellet was washed with 1 ml of ice-cold saline and resuspended in 1 ml of ice-cold 10% trichloroacetic acid. After 10 min, the precipitate was washed once with1 ml ice-cold trichloroacetic acid and then counted in 5 ml of scintillation fluid.

RESULTS AND DISCUSSION

Hexose Monophosphate Pathway (HMP)

The reductive activation of MISO by hypoxic EMT6/Ro and wildtype Chinese hamster ovary (CHO) cells, as indicated by the binding of ^{14}C-MISO to the acid-insoluble fraction of these cells, was increased by the presence of glucose (Table I). Further, determination of the clonogenic survival after incubation of hypoxic EMT6/Ro tumor cells with MISO revealed that cells incubated in the presence of glucose appear to be more sensitive to MISO than those cells incubated without glucose.[13]

In a variety of cells the HMP is considered the major pathway for providing reducing equivalents in the form of NADPH for reductive metabolism. In line with

TABLE I

Binding of ^{14}C-misonidazole to the acid-insoluble fraction of EMT6/Ro, wildtype CHO and mutant CHO cells deficient in glucose-6-phosphate dehydrogenase in the presence and absence of glucose

Cell	Binding of ^{14}C-MISO (nmol/10^6 cells × 3 h)	
	6 mM glucose	without glucose
EMT6/Ro	7.0 ± 0.4	3.0 ± 0.3
CHO wildtype	4.3 ± 0.3	2.6 ± 0.3
CHO mutant	4.2 ± 0.2	2.8 ± 0.3

Incubations were performed under hypoxic conditions at 37°C for 3 h. The concentration of ^{14}C-misonidazole (MISO) was 5 mM. SE for the mean for 3 incubations are given.

results obtained by Varnes *et al.*[14] in Ehrlich and A 549 cells these was a marked stimulation of the activity of the HMP following addition of MISO to hypoxic EMT6/Ro and wildtype CHO cells (Table II). Moreover, there is a rough correlation between the stimulation of the HMP and the hypoxic toxicity of MISO in both cells lines used here (compare Fig. 2, Table II). These data together with the glucose effects on the activation of MISO and its hypoxic toxicity (see above) point to glucose as the principle source of electrons and to the HMP as the principle pathway for providing reducing equivalents for the activation of MISO.

Glycolysis

Under the assumption that an active HMP is required for the supply of reducing equivalents for the activation of MISO one should expect a marked decrease in the activation of MISO and hence in its hypoxic toxicity in cells without active HMP. However, comparing the hypoxic activation of MISO and its hypoxic toxicity in mutant CHO cells deficient in glucose-6-phosphate dehydrogenase and therefore without active HMP (Table II) with their parent wildtype CHO cells revealed only a somewhat lower sensitivity of the mutant cells (Fig. 2) with almost no difference in the binding of activated MISO to the acid-insoluble fraction of these cells (Table I). These results suggest that reducing equivalents for the activation of MISO can also be provided by metabolic pathways other than the HMP. Since in CHO cells as well as EMT6/Ro cells a decrease in the binding of activated MISO was observed in the absence of glucose (Table I), glycolysis is another possible pathway providing electrons for the activation of MISO. Contribution of glycolysis and thus of NADH to

TABLE II

Activity of the hexose monophosphate pathway in EMT6/Ro, wildtype CHO and mutant CHO cells deficient in glucose-6-phosphate dehydrogenase in the absence and presence of misonidazole

Cell	$^{14}CO_2$ formation (nmol/10^7 cells × h)	
	without MISO	5 mMISO
EMT6/Ro	85 ± 6	213 ± 11
CHO wildtype	32 ± 3	65 ± 4
CHO mutant	< 0.1	< 0.1

The activity of the hexose monophosphate pathway was estimated by the determination of the formation of $^{14}CO_2$ from 6 mM ^{14}C-1-glucose under hypoxic conditions. MISO, misonidazole. Further experimental details are as indicated in Table I.

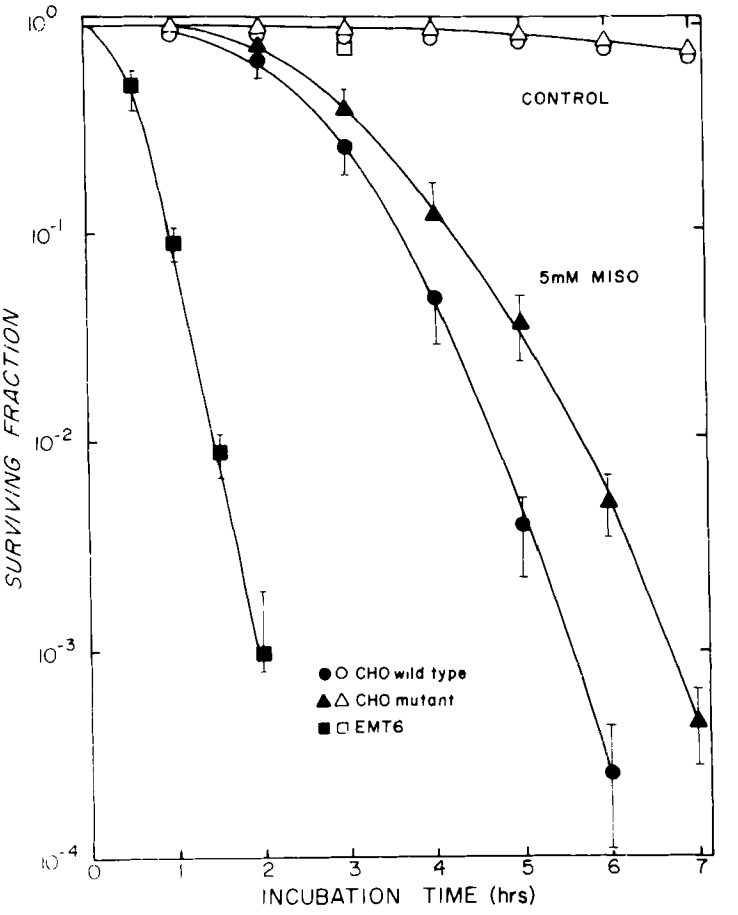

FIGURE 2 Clonogenic survival of EMT6/Ro, wildtype CHO and mutant CHO cells deficient in glucose-6-phosphate dehydrogenase exposed to 5 mM misonidazole (MISO) under hypoxic conditions. SE of the mean for 3 incubations are given.

the reductive activation of MISO should be energetically favoured by an increase in the NADH/NAD ratio with increasing hypoxia.

Besides their possible role for providing reducing equivalents for the activation of MISO, glycolytic enzymes appear to be one of the targets of the reactive MISO metabolites. So for example, in the case of the EMT6/Ro cells even 1 h after the addition of MISO, there is a marked inhibition of glycolysis as indicated by glucose consumption and lactate formation.[15] At least partial inhibition of glycolysis has also been described for a variety of other cells.[16] It may be due to reaction of reactive MISO metabolites with essential thiol groups of glycolytic enzymes.[17] Since glycolysis is the major ATP source of the hypoxic cell, inhibition of glycolysis may contribute to the hypoxic toxicity of MISO. On the other hand, at relatively low MISO concentrations (e.g. 1 mM) a decrease in clonogenicity had occurred before there was a decrease in anaerobic glycolysis detectable.[15]

DISCUSSION

A prerequisite for the hypoxic toxicity of MISO is its reductive activation to reactive products such as the nitro radical anion, the nitroso and the hydroxylamine derivative. The reductive activation is inhibited by oxygen. The data presented here indicate that not only the HMP but also glycolysis may function as electron sources for the activation of MISO. In addition, glucose-independent pathways appear to contribute an essential portion of reducing equivalents to the reductive activation of MISO. As shown in Table I, the residual binding of activated MISO occurring in the almost complete absence of glucose can account for up to 67% of that binding observable in the presence of glucose. One of these glucose-independent pathways may start with the formation of NAD(P)H by mitochondrial glutamate dehydrogenase and may involve mitochondrial di- and tricarboxylate carriers as well as mitochondrial and cytosolic isocitrate dehydrogenases (see 18 for more detailed information).

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